Arch Dermatol Res (2001) 293 : 291–295

ORIGINAL PAPER

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Effect of nerve growth factor on endothelial cell biology: proliferation and adherence molecule expression on human dermal microvascular endothelial cells

Received: 7 December 2000 / Revised: 10 December 2000 / Accepted: 24 February 2001

Abstract **In addition to its effect on the central nervous system, nerve growth factor (NGF) appears to play a key role in the initiation and maintenance of inflammation in many organs. NGF degranulates mast cells, recruits inflammatory cellular infiltrates and activates T cells. Extravascular migration of leukocytes is initially controlled by the interaction of cell surface adhesion molecules of leukocytes and endothelial cells. A marked upregulation of NGF in keratinocytes is also observed in conditions characterized by angiogenesis such as psoriasis and wound healing. In this study we investigated the role of NGF in inflammation by studying its effects on endothelial cell proliferation and intracellular adhesion molecule expression by endothelial cells. The effect of NGF on human dermal microvascular endothelial cell (HDMEC) proliferation was measured using the hexosaminidase assay. ICAM-1 expression on HDMEC was measured by ELISA. The function of ICAM-1 was assessed by adherence of peripheral blood mononuclear cells (PBMC) to HDMEC using ^{51}Cr-labeled PBMC. There was a significant increase in proliferation of HDMEC stimulated with NGF as compared to unstimulated HDMEC ($P < 0.001$). NGF-neutralizing antibody decreased the mitogenic effect of NGF significantly ($P < 0.05$). NGF also increased ICAM expression on HDMEC as compared to unstimulated HDMEC ($P < 0.05$). NGF-neutralizing antibody decreased ICAM expression on NGF-stimulated HDMEC ($P < 0.05$). The percentage of PBMC adherence was higher in NGF-stimulated HDMEC ($P < 0.001$). Anti-ICAM antibody decreased PBMC adherence. In the study reported here, the role of NGF in two important aspects of inflammation, i.e. angiogenesis and inflammatory cell recruitment at the site of inflammation, was investigated**.

Keywords NGF · Endothelial cell · Adhesion molecule · Inflammation

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Introduction

Nerve growth factor (NGF) was discovered nearly five decades ago [1]. It is a neurotrophic factor, mainly responsible for survival and development of a specific population of neurons in the peripheral and central nervous systems. In recent years several investigators have found that NGF and its receptor system (NGF-R) have wider biological activities than thought previously. Observations have suggested a regulatory role of NGF/NGF-R systems in the inflammatory cascades [2–4]. Injection of NGF in nanomolar concentrations induces the influx of inflammatory infiltrates. NGF is chemotactic to leukocytes, and upregulation of NGF levels is observed at sites of inflammation, e.g. wounds, psoriatic plaques and the synovium of inflamed joints [5–8]. The Expression of adhesion molecules on the endothelial cells and formation of new blood vessels are integral components of an inflammatory reaction. The role of NGF on endothelial cell biology is an unaddressed issue. In this study we investigated the effects of NGF on endothelial cell proliferation and expression of ICAM-1.

Materials and methods

Human dermal microvascular endothelial cell proliferation

Adult human dermal microvascular endothelial cell (HDMEC) from pooled donors were used in the third passage (Clonetics, San Diego, Calif.). The culture medium was EGM-MV without hydrocortisone (Clonetics, San Diego, Calif.). Endothelial cell proliferation was assessed using the hexosaminidase assay. The substrate for hexosaminidase was *p*-nitrophenol-*N*-acetyl-β-D-glucosaminide (Sigma, St. Louis, Mo.). Human recombinant TNF-α (hrTNF-α) (Boehringer Mannheim, Indianapolis, Ind.), IFN-γ (Endogen, Woburn, Mass.)

and NGF-β (Boehringer Mannheim) were used as stimulants. hrTNF-α and IFN-γ were used as positive controls for the proliferation assays. NGF-β was used at several concentrations, i.e. 10, 100 and 1000 ng/ml. NGF-neutralizing antibody (100 ng/ml) was used to assess the inhibition of NGF-induced HDMEC proliferation.

Cells were plated at 3300 per well in flat bottomed 96-well microtiter plates (Corning, N.Y.). After 24 h, TNF-α, IFN-γ, NGF-β and NGF+NGF-neutralizing antibody were added to activate the cells in triplicate wells. The cells were incubated for 2 and 5 days. The substrate solution was added in volumes of 60 µl to cells in the microtiter wells. The plates were then incubated at 37 °C in 100% humidity. After a suitable interval, the color reaction was developed and enzyme activity blocked by the addition of 50 m*M* glycine buffer, pH 10.4, containing 5 m*M* EDTA, 90 µl per well. Absorbances were measured in an ELISA reader at 405 nm.

ICAM-1 expression

HDMEC (2×10^4 cells/well) were plated in 24-well plates (Falcon, BD, N.J.) pretreated with attachment factor (Cascade Biologics, Portland, Ore.) for 24 h. The medium (EGM-MV; Clonetics) contained 20% fetal calf serum (FCS). Each experiment was done in triplicate. After 24 h, the medium was changed to EGM-MV containing 5% FCS and the following reagents were added separately: NGF-β (10, 100, and 1000 ng/ml), NGF-β with anti-NGF antibody, TNF-α (5, 10, and 20 ng/ml), substance P (SP, 10^{-7} and 10^{-8} *M*). Three wells were kept with medium only as control. After a 24-h incubation, the cells were washed three times with PBS. The cells were fixed with 0.5% glutaraldehyde for 10 min. At 4 °C, cells were washed three times with PBS containing 5 m*M* EDTA and 0.1% bovine serum albumin (BSA). Then ICAM-1 monoclonal antibody at a concentration of 0.5 µg/ml was added to the wells. After 1 h, the wells were rinsed three times with PBS containing 0.1% BSA and 100 µl/well goat anti-mouse IgG antibody labeled with peroxidase (1:1000 in HBSS, i.e. 0.1% BSA) was added (Biorad, Hercules, Calif.). After 1 h, the wells were washed and TMB solution (100 µl/well; Sigma) was added to each well. After 30 min H_2SO_4 (0.5 *M*, 50 µl/well) was added and mixed. A 100-µl aliquot of the mixture was transferred to 96-well plates and read at 492 nm.

Function of ICAM-1 assessed by adhesion of ^{51}Cr-labeled peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-hypaque (Pharmacia, Uppsala, Sweden) gradient centrifugation from five normal individuals. HDMEC were distributed to attachment factor-coated flat bottomed 96-well plates (1.5×10^4 cells per well in 100 µl medium). After overnight incubation, wells were washed gently with medium. To each well, NGF-β (10, 100, 1000 ng/ml), TNF-α (5,10, and 20 ng/ml), SP (10^{-7} and 10^{-8} *M*) and medium only were added separately. After 48 h the wells were washed with medium and 50 µl ^{51}Cr-labeled normal PBMC suspension (containing 75,000 cells) was added. Plates were incubated for 90 min at 37 °C in an atmosphere containing 5% CO_2. Then the wells were gently washed to remove nonadherent PBMC. Remaining cells (HDMEC and adherent PBMC) were lysed with 100 µl 1% Triton-X in PBS. Lysates were removed and counted in a gamma counter.

Adherence was determined as the percentage of the total radioactivity added to the wells (adherence ratio). All normal PBMC were tested in triplicate. To obtain a background count, three wells were processed in the same way but without added PBMC.

NGF-stimulated HDMEC, NGF with anti-NGF antibody-stimulated HDMEC and normal PBMC were pretreated with monoclonal antibodies for 15 min, following which the adhesion assay described above was performed in the presence of the following monoclonal antibodies: anti-ICAM-1 (CD54) (10 µg/ml), anti-CD11a (20 µg/ml), and anti-CD18 (10 µg/ml) (Endogen, Woburn, Mass.). Adhesion inhibition was calculated by the formula of Vennegor et al. [9].

Results

Effect of NGF on HDMEC proliferation

Figure 1 shows that NGF increased HDMEC proliferation ($P < 0.001$, Student's *t*-test). We evaluated the dose response curve using different concentrations of NGF. The highest response was seen at 100 ng/ml (Fig. 2). NGF-neutralizing antibody decreased NGF-induced HDMEC proliferation ($P < 0.01$, Student's *t*-test; Fig. 1). TNF-α produced an increase in HDMEC proliferation ($P < 0.001$; Fig. 1). TNF-α is a known inducer of endothelial cell proliferation. Thus, it validates the use of the hexosaminidase assay to assess HDMEC proliferation. IFN-γ also produced an increase in HDMEC proliferation as compared to medium alone ($P < 0.05$; Fig. 1). The dose response curve for TNF-α and IFN-γ was also evaluated (data not shown). These results suggest that NGF plays an important in angiogenesis.

Effect of NGF on ICAM-1 expression

Figure 3 shows an increase in ICAM expression by endothelial cells stimulated with NGF at 100 ng/ml ($P < 0.01$,

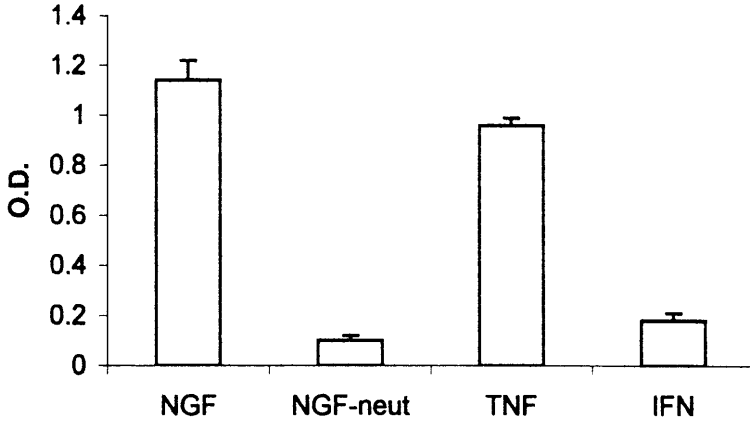

Fig. 1 Effects of NGF on endothelial cell proliferation. NGF (100 ng/ml) induced endothelial cell proliferation. NGF-neutralizing antibody (100 ng/ml) inhibited NGF-induced cell proliferation (*NGF-neut*). Each bar represents the mean ± SEM of ten experiments. TNF-α and IFN-γ were used as positive control (*TNF* TNF-α 10 ng/ml, *IFN* IFN-γ 50 ng/ml)

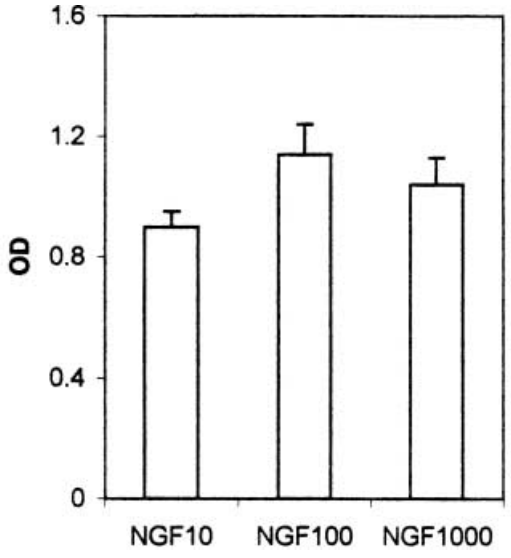

Fig. 2 Dose response curve of proliferation of endothelial cells induced by NGF at 10, 100 and 1000 ng/ml

Student's *t*-test). The neuropeptide SP also increased ICAM expression by endothelial cells ($P < 0.001$). TNF-α is a known ICAM-inducing agent and it increased ICAM expression by endothelial cells ($P < 0.001$). The dose response curve for TNF-α and SP was also evaluated (data not shown).

Effect of NGF on ICAM-1 function

Figure 4 shows the functionality of ICAM expressed on HDMEC. ICAM induced by NGF ($P < 0.01$), NGF+anti-NGF antibody, TNF-α ($P < 0.001$) and SP ($P < 0.01$, Student's *t*-test) produced an increase in PBMC adherence to HDMEC as compared to medium only. Table 1 shows the inhibitory effect of anti-ICAM antibody (CD54) on PBMC adhesion. Both CD11a and CD18 antibody also resulted in inhibition of the ^{51}Cr count, an indicator of PBMC adhesion to HDMEC in this experimental model. Each experiment was carried out ten times.

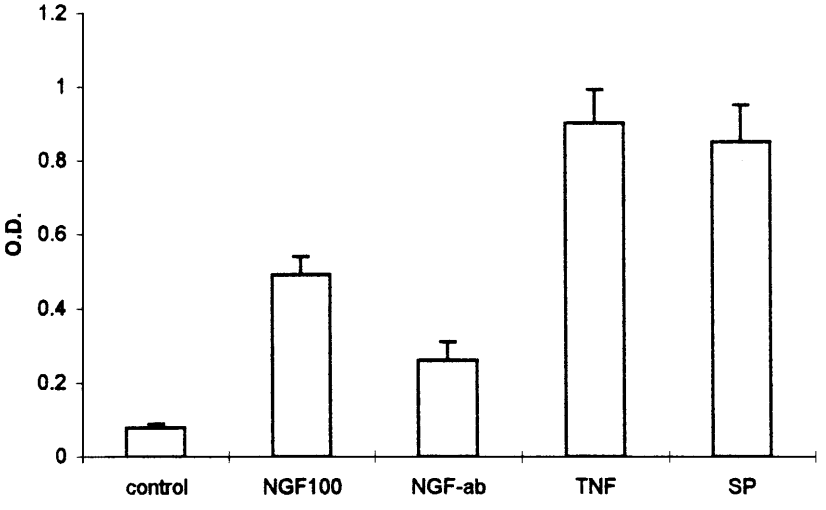

Fig. 3 NGF induces ICAM-1 expression on endothelial cells (*NGF100* NGF at 100 ng/ml, *NGF-ab* NGF+anti-NGF antibody, *TNF* TNF-α 10 ng/ml, *SP* substance P 10^{-8} *M*). TNF-α and SP were used as positive controls. Each bar represents the mean ± SEM of ten experiments

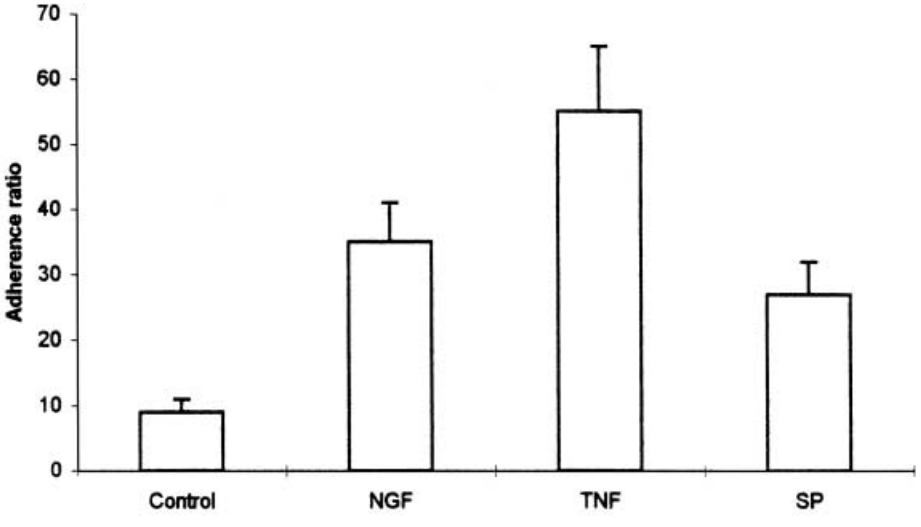

Fig. 4 PBMC adhered to HDMEC stimulated with NGF, NGF+anti-NGF antibody, TNF-α and SP. The results are expressed as the means ± SEM of ten experiments

Table 1 Inhibition of PBMC adhesion to NGF-stimulated endothelial cells by monoclonal antibodies. The adhesion inhibition was determined using the ^{51}Cr assay and is expressed as a percentage taking medium only as 100%. PBMC did not adhere to the NGF+anti-NGF antibody-stimulated endothelial cells

Antibody	Percentage inhibition (mean ± SEM)	No. of experiments
CD11a	47 ± 3	10
CD18	58 ± 5	10
CD54	65 ± 6	10
Control	1.4 ± 0.2	10

Discussion

Several in vivo and in vitro studies have shown that NGF can induce an inflammatory response. NGF upregulates neuropeptides [10, 11], degranulates mast cells [12], induces expression of RANTES in keratinocytes [13], activates T cells and causes extravasation of leukocytes [5, 14–16]. In this study we observed two additional functions of NGF which are key components of an inflammatory response: upregulation of ICAM-1 and proliferation of endothelial cells.

Leukocyte trafficking under normal and diseased conditions is generated by expression of adhesion molecules, chemokines and other chemotactic compounds [17–19]. Endothelial cells play important roles in this process, and binding of leukocytes to endothelial cells is the first essential step. Microvascular endothelial cells of human skin contribute to the recruitment of inflammatory leukocytes by expressing inducible leukocyte adhesion molecules such as endothelial leukocyte adhesion molecule-1 (ELAM-1 or E selectin), vascular cell adhesion molecule-1 (VCAM-1), and ICAM-1. Increased expression of ICAM-1 is closely associated with T-cell migration in vivo but also contributes to adhesion of granulocytes [20–22]. Leukocytes bind to ICAM-1 on the endothelial cell surface via its ligand leukocyte function-associated-1 molecule (LFA-1 or CD11a/CD18).

Expression of ICAM-1 is restricted on resting cells but is highly inducible by exposure to activators such as IL-1 β or IFN-α [23, 24]. Here we observed that NGF is a potent inducer of ICAM-1 on HDMEC. Further, the use ^{51}Cr-labeled PBMC demonstrated a marked increase in leukocyte adherence on NGF-stimulated HDMEC compared to the controls ($P < 0.001$). There was no increase in PBMC adherence when HDMEC were stimulated with NGF+anti-NGF antibody. This determines the specificity of NGF stimulation of HDMEC. In chronic inflammatory conditions such as psoriasis and rheumatoid arthritis, marked upregulation of ICAM-1 on vessels is associated with increased levels of NGF at the sites of inflammation [7, 8]. Rapid recruitment of leukocytes following subdermal injection of NGF in mice has been reported [5]. The molecular mechanisms involved in NGF-induced leukocyte migration in vivo are not completely understood. Our observations in this study suggest that the induction of ICAM-1 by NGF plays a role in cell trafficking and perpetuation of a chronic inflammatory process. However, in addition to induction of ICAM-1, NGF can promote an inflammatory reaction by degranulating mast cells [12].

In this study we observed another novel function of NGF on endothelial cell biology: it was mitogenic to the HDMEC. The physiological significance of NGF being mitogenic to endothelial cells is unknown. However, in several pathological states, such as healing wounds, psoriasis and rheumatoid arthritis angiogenesis, is a key histological feature. Increased levels of NGF have also been reported to be associated with these conditions [6–8]. It is likely that NGF plays a role in promoting and maintaining the processes involved in neovascularization in these conditions. NGF induces proliferation of neural cells (PC12 cell line) and keratinocytes [25, 26] via the c-*fos* gene activation pathway. The c-*fos* gene also stimulates vascular endothelial cell proliferation [27, 28]. Thus, it appears that NGF may increase endothelial cell proliferation via the c-*fos* gene pathway.

A number of studies have indicated that NGF plays a role in cutaneous wound healing: intradermal injection of NGF increases the rate of sprouting in denervated skin [29]; NGF mRNA and protein have been detected in the keratinocytes of the growing edge of a wound [3]; NGF is mitogenic to keratinocytes [30]; and application of NGF to a wound increases the rate of reepithelialization [6]. Angiogenesis is an essential component of the granulation tissue in healing wounds. Proliferation of endothelial cells induced by NGF provides a new insight into the role of NGF in the formation of granulation tissue, an absolute requirement for the healing of a wound. Nature uses its resources very efficiently and economically. It is possible that NGF may play a role in several key events in wound healing such as reepithelialization, reinervation and angiogenesis.

Acknowledgements This work was supported by a Psoriasis Research Institute endowment.

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